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(57) Abstract

The invention relates to specific bacterium and proteins with xylanase activity derived from the bacteria, in particular to xylanases which are free of any significant cellulase activity and which are active at high temperature and at neutral to alkaline pH. Xylanases having these characteristics are particularly useful in the bleaching of wood pulps, such as kraft pulps. The preferred bacterium designated B230 was isolated from white-rotted kerri wood in Western Australia; a sample of which has been deposited under the provision of the Budapest Treaty in the Australian Government Analytical Laboratories under the accession number N94/41262. This preferred bacterium is a gram positive, obligatively aerobic, rod-shaped with a centrally-located spore and has the taxomonic characteristics of *Bacillus subtilis* (by VITEK method).

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BACTERIAL PROTEIN WITH XYLANASE ACTIVITY

This invention relates to proteins with xylanase activity derived from bacteria, and in particular to xylanases which are free of any significant cellulase activity and which are active at high temperature and at neutral to alkaline pH. Xylanases having these characteristics are particularly useful in the bleaching of wood pulps, such as kraft pulps.

BACKGROUND OF THE INVENTION

Enzymes are proteins present in all living cells, where apart from controlling metabolic processes, they break down food materials into simpler compounds. The enzymes are catalysts which speed up processes which would otherwise proceed very slowly, or not at all. Moreover, enzymes are very specific, breaking down only one type of compound.

Xylan is a polysaccharide found in most plant cell walls, consisting of D-xylose units linked by β-1-4 glycosidic bonds. It occurs with another polysaccharide, cellulose and an amorphous binding polymer, lignin. Xylan forms a major component of plant hemicelluloses, and varies in the nature of substituents on the sugar groups, depending on the origin. For example, xylans derived from hardwoods typically consist of a backbone of O-acetyl-4-O-methylglucuronoxylan, in which about 10% of the xylose units carry 4-O-methylglucuronic acid side chains linked via α-1,2 bonds, and 70% of the xylose residues are acetylated at C-2 or C-3. In contrast, xylans derived from softwoods are usually arabino-4-O-methylglucuronoxylans in which over 10% of the xylose sub-units carry arabinofuranose residues linked via α-1,3 bonds. Enzymes which are able to degrade xylan are called xylanases (endo-1,4-β-D-xylanases; International enzyme nomenclature EC 3.2.1.8).

Commercial preparations of xylanase, often in combination with other cell wall degrading enzymes, have been used in the extraction or liquefaction of plant material. For example, in the food industry, the mashing process for the production of juices can be made to produce higher yields and better processing with the application of cell wall degrading enzymes, which include xylanase.

The primary source of cellulose for paper manufacture is wood, and may be either hardwood or softwood. The initial step in paper manufacture is the reduction of wood to the fibre state, which may be achieved by mechanical or chemical pulping methods. Chemical pulping involves the "cooking" of woodchips with chemical reagents in order to separate the cellulose fibres from the other wood components, and to break down the lignin and other extraneous compounds so that the cellulose is left in tact in its fibrous form. The most common process is the kraft or sulphate process, which can be applied to almost any timber species. The active ingredients are sodium hydroxide and sodium sulphide in a strongly alkaline solution.

During the kraft pulping process, xylan in the wood is initially dissolved in the pulping liquor, but with time, reprecipitates on to the resulting pulp. Wood lignin is modified and dissolved by the pulping liquors. However, about 10% of the lignin remains in the kraft pulp. To brighten the pulp, the lignin must be removed by bleaching chemicals, such as chlorine, which generate environmentally hazardous wastes.

More recently, commercial xylanase preparations have been used as an aid to the bleaching of kraft wood pulps. A program of cooperation between research institutes and the pulping industry has shown that treating the unbleached kraft pulp with xylanase results in a reduction in the amount of bleaching chemicals required to obtain a full brightness pulp. It is believed that xylanase acts as a bleaching aid (bleach booster) by

releasing some trapped residual lignin within the pulp matrix and giving better access to bleaching chemicals. It is widely believed that xylanase breaks down reprecipitated xylan which forms a coating on the pulp, thus releasing trapped residual lignin from within the pulp matrix, and allowing better access of bleaching chemicals to this matrix. Thus xylanase acts as a bleaching aid or bleach booster.

In the kraft process, the pulp is typically handled at high temperatures and neutral to alkaline pH. Commercial xylanases typically have a temperature optimum of about 50°C and a pH optimum of about 5, and are thus subject to rapid denaturation under process conditions. Thus there is a need for xylanases which are able to act optimally on the kraft pulp without any requirement to adjust the temperature or pH. In order to be useful as a bleaching aid, the xylanase must also be free of any significant cellulase activity, since cellulase would cause an undesirable loss of cellulose fibre.

We have screened microorganisms newly isolated from a range of environments in order to identify those which produce high levels of xylanases with high temperature optima and which are active at neutral to alkaline pH. A previously unidentified bacterium isolated from white-rotted wood, produces such a xylanase in high yield and free of significant cellulase activity. Thus bacterium is a strain of *Bacillus Subtilis* which we have designated B230.

Summary of the Invention

According to one aspect, the invention provides a bacterium, isolatable from wood compost, having the following characteristics:

A. Ability to grow at a temperature between 20° and 45°;

B. Ability to grow in the pH range of 5 to 9.5;

C. Ability to grow on Luria-Bertani agar at 37°;

D. Ability to grow under solid state or submerged culture conditions; and

E. Constitutive production and/or extracellular release of at least one protein with xylanase activity having an associated cellulase activity of less than 0.1, said at least one protein having a molecular weight of about 28kD.

Preferably the bacterium is isolated such that a biologically pure culture exits.

Preferably xylanase production is enhanced by growth in the presence of xylan or of lignocellulose substrates, or degradation products, including xylose and xylitol, derived from such substrates.

More preferably the xylanase has at least one characteristic selected from the group consisting of activity at about pH between 4.5 and 9.5, a thermal activity range up to 70°C, and high thermal stability up to 65°C. Most preferably the xylanase produced by the bacterium is effective on both soluble and insoluble xylans.

In a particularly preferred embodiment, the bacterium has the characteristics of the bacterial isolate designated B230, as deposited under the provisions of the Budapest Treaty in the Australian Government Analytical Laboratories, PO Box 385, Pymble, New South Wales 2073, Australia, on 6 September 1994, under Accession No. N94/41262, or a mutant or derivative thereof having the ability to produce a xylanase as described above. The term "mutant or derivative" thereof includes naturally occurring and artificially induced mutants which retain their ability to digest xylans. Production of such mutants or derivatives will be well known by those skilled in the art.

According to a second aspect, the invention provides a process for producing at least one protein with

xylanase activity said process comprising cultivating a bacterium under conditions and for a time sufficient to produce said protein and collecting culture medium wherein said bacterium has the following characteristics:

A. Ability to grow at a temperature between 20 and 45°;

B. Ability to grow in the pH range of 5 to 9.5;

C. Ability to grow on Luria-Bertani agar at 37°;

D. Ability to grow under solid state or submerged culture conditions; and

E. Constitutive production and/or extracellular release of at least one protein with xylanase activity, said protein having an associated cellulase activity of <0.1%.

Preferably the bacterium used is strain B320 or a mutant, variant or derivative thereof.

Preferably the bacterium is grown under optimal conditions for extracellular production of said at least one protein. Still more preferably the production of said at least one protein is induced by the addition of xylitol to the culture medium. Preferably xylitol is added in an amount of 0.01 to 2% of the culture medium which is preferably a broth.

According to a third aspect, the invention provides a protein with xylanase activity said protein having an associated cellulase activity of less than 0.1% and a molecular weight of about 28kD as determined by SDS-PAGE. Preferably the protein has at least one characteristic selected from the group consisting of activity at about pH between 4.5 and 9.5, a thermal activity range up to 70°C and high thermal stability up to 65°C. Preferably the protein is effective in digesting both soluble and insoluble xylans.

Preferably the protein with xylanase activity is isolatable from the bacterium described above. More

preferably the protein is isolated from the bacterial strain B230.

Preferably the protein with xylanase activity is an isolated preparation meaning that it has undergone some purification away from other proteins and/or non-proteinatious material. The purity of the preparation may be represented as at least 40% protein with xylanase activity, preferably at least 60% protein, more preferably at least 75% protein with xylanase activity, still more preferably at least 80% protein with xylanase activity or greater, as determined by weight, activity, amino acid composition or similarity, antibody reactivity or any other convenient means.

According to a fourth aspect, the invention provides a composition comprising said protein with xylanase activity as an active ingredient together with an industrially acceptable stabiliser. The composition may be used as a bleaching aid or bleaching booster or in paper deinking. Those skilled in the art will be familiar with the types of industrially acceptable stabilisers which may be used such as glycerol, sorbitol or other polyalcohols.

The composition described above is for use in bleaching kraft pulp or deinking paper. Accordingly, in a fifth aspect the present invention provides a method of bleaching wood or paper pulp comprising administering a bleaching aid or bleaching booster effective amount of the composition to said pulp, for a time and under conditions sufficient to achieve the desired bleaching of the pulp.

The protein of the present invention may also be used in the preparation of animal feed and in preparation of dough for bread-making.

We have found that the bacterium B230, when grown under suitable fermentation conditions, will produce xylanase which accumulates in the extracellular fermentation broth. The xylanase from such a broth has a thermal activity range from ambient up to 70°C and a useful pH range from 5 to 9, with optimal activity at pH 6 - 6.5.

The xylanase has very high thermal stability, retaining 100% activity after 3 hrs and 90% activity after 22 hrs at 60°C. Cellulase activity associated with the xylanase is minimal (<0.1%).

The crude preparation may be used however partially purified xylanase may also be used.

While the following description refers to a single xylanase, our results indicate that there are in fact at least two different xylanases produced during fermentation of bacterium B230, and all xylanases produced by this organism are within the scope of the invention.

Description of the Invention

The invention will now be described by way of reference only to the following non-limiting examples, and to the figures, in which:

Figure 1 shows the variation of activity of xylanase from bacterium B230 with pH compared with that from bacterium B698, and

Figure 2 illustrates the variation in activity of xylanase from bacterium B230 with temperature, compared with that from bacterium B698.

Figure 3 is a photograph of a SDS-PAGE gel of the purified enzyme having an approximate molecular weight of 28kD.

Figure 4 is a photograph of a SDS-PAGE gel of fermenter broth proteins including xylitol induced xylanase. Compared with proteins from non-induced cultures, the xylanase protein can be identified as having an approximate molecular weight of 28kD.

Figure 5 illustrates the colour units release by xylanase from bacterium B230 at a range of pH and temperatures.

Example 1

A bacterium which we have designated B230 was isolated from a sample of white-rotted karri wood; this sample was collected from near Walpole, Western Australia,

in May 1993.

Approximately 0.5g of sample was placed in a 25mL conical flask. To this was added 10mL of sterile deionised water, and the flask was placed on an orbital shaker at room temperature for 30 minutes. Serial dilutions of the water dispersion were prepared as follows:

0.9mL of sterile water was added into four 1mL sterile tubes. A sample of water (0.1mL) from the 10mL flask was added to the first tube. The contents of the tube were mixed well, and 0.1mL added to the second tube, and the procedure was repeated down to the fourth tube.

Samples (0.1 mL) from each tube was streaked onto Luria-Bertani agar. The agar plates were sealed and placed in a incubator at 37°C overnight. Colonies of bacteria appeared on the plates, and individual colonies were picked off and replated onto fresh Luria-Bertani plates.

The composition of Luria-Bertani medium is:

tryptone 10g
yeast extract 5g
sodium chloride 10g
deionised water 1L

For Luria-Bertani (LB) agar, 18g of agar is added to the above components. All media were sterilised by autoclaving at 121°C for 20 minutes.

The organism was isolated in pure culture, and a sample was deposited under the Budapest Treaty in the Australian Government Analytical Laboratories as described above.

The bacterium has the following taxonomic characteristics:

rod-shaped bacterium with a centrally-located spore
Gram positive
obligately aerobic
Bacillus subtilis (by VITEK method)

Example 2 Growth Conditions

The bacterium is not fastidious, and can be grown on a range of media, including LB broth. The requirements are:

1. a source of carbon, most conveniently a carbohydrate such as dextrose,
2. a source of nitrogen, most conveniently as a tryptone, and
3. complex nutrients, most conveniently as yeast extract.

The bacterium can be grown within the temperature range 20 to 45°C and within the pH range 5 to 9.5.

The bacterium can be grown successfully under different fermentation conditions, including solid state or submerged culture; fermentation continues under aerobic conditions with or without agitation.

Example 3 Production and Characterisation of Xylanase

When grown under the conditions described in Example 2, bacterium B230 synthesises xylanase, and releases the enzyme into the extracellular medium. While xylanase is produced constitutively, addition of xylan to the culture medium as an additional carbon source further enhances the level of xylanase production. The added xylan may be in the form of isolated wood xylan, or may be a component of lignocellulosic material such as wheat bran.

Xylanase was assayed using the following conditions:

Substrate: 1% birchwood xylan
Buffer: 50mM sodium phosphate/citric acid, pH 6.
Incubation temperature: 50°C
Incubation time: 20 minutes

The enzyme reaction was stopped with 3,5-dinitrosalicylic acid (DNS) reagent which measures, using xylose standards, the amount of reducing sugar produced in 20 minutes. Enzyme units are expressed in nanokatals

(nkats), where 1 nkat is the amount of xylanase which will produce 1 nmole of xylose per second under the defined conditions.

Example 4 Production of Xylanase by Submerged Fermentation

Xylanase from B230 can conveniently be prepared by submerged fermentation. B230 seed culture can be prepared overnight in LB broth at 37°C. This inoculum is added to an LB broth containing beechwood xylan (2% w/v). The pH of the broth is increased to pH 7.8 by the addition of 2M sodium hydroxide, and the temperature adjusted to 37°C. The broth is stirred (1,000 rpm) and aerated with filtered sterile air (0.7 L of air/L of broth/min).

The seed inoculum is added to the broth and the above conditions of temperature, pH, agitation and aeration maintained. Samples of culture are taken at regular intervals to monitor the production of xylanase. Optimal levels of xylanase (11,000 nkat/mL) are obtained within 90 hours of fermentation.

Example 5 Characterisation of Xylanase

The crude enzyme preparation from the fermenter broth was characterised with respect to pH and temperature.

a) pH Optimum

The xylanase activity was determined as described above, with the exception that the buffer was changed to obtain a stable pH. The results are listed in Table 1 below. The data is further expressed in Figure 1. The optimal pH for xylanase activity was found to be pH 6-6.5.

Table 1

pH Profile of B230 Xylanase

pH	Relative Xylanase Activity (%)
4	24
5	87
6	100
7	72
8	76
9	32
10	11

b) Temperature Optimum

The xylanase activity of B230 enzyme was determined as described above, except that the temperature was altered within the range from 40 to 80°C. Results are listed in Table 2 and further expressed in Figure 2. The optimal temperature for xylanase activity was found to be 60°C.

Table 2

Temperature Profile of B230 Xylanase

Temperature (°C)	Relative Xylanase Activity (%)
40	45
50	63
60	100
65	80
70	42
80	8

Example 6 Thermal Stability

The stability of B230 xylanase was determined at pH 6 and 60°C, the optimal pH and temperature respectively for the enzyme system. Samples were tested for residual

activity at regular intervals as described in the xylanase assay conditions above. After 3 hours, 100% xylanase activity was retained. Even after 22 hours, 90% of the xylanase activity was retained. Thus, xylanase from B230 is very thermally stable.

The thermal stability at 60°C and 65°C at different pH values were determined over 2 hours. Results are in Table 3.

Table 3
Thermal Stability 60, 65°C, 2 hrs

pH	Relative Xylanase Activity 60°C	65°C
6	100	71
7	117	48
8	84	7
9	55	0

Example 7 Stability at 4°C

The stability of B230 xylanase was determined at 4°C by storing it at that temperature. Samples were tested for activity at regular intervals under the conditions described in the xylanase assay conditions above. After 22 days, 100% of the original activity was retained.

Example 8 Purification of Xylanase

A fraction of xylanases was partially purified by conventional purification techniques involving DEAE Sepharose and size exclusion chromatography. The xylanase fraction had a single band on SDS-PAGE at 28kDa as shown in Figure 3 and a purity of > 80%.

Example 9 Induction of 28kDa Xylanase

B230 seed culture was prepared overnight in LB broth at 37°. This inoculum was added equally to 2 flasks containing corn steep liquor (2%) and incubated at 37°C. To one flask, xylitol (to 0.1%) was added daily for 5 days. After 5 days both flask broths were centrifuged. The cell free broths were assayed for xylanase activity. Xylitol

induces xylanase (2,000nkat/ml) compared with uninduced broth (50nkat/ml). A sample of each broth was concentrated by ultrafiltration (5kDa membrane), and the retentate run on an SDS-PAGE gel. As shown in figure 4, a protein band at approximately 28kDa was induced by xylitol. This is consistent with the purified xylanase in Example 8, figure 3.

Example 10

Use Of B230 Xylanase As A Bleaching Aid

The crude xylanase system (167nkat/g of pulp) was mixed with unbleached kraft pulp (35 g oven dried basis) at consistency 8% and adjusted to pH 5, 7 or 9 with appropriate buffer. The mixture was incubated for 1 hr at 60°C. The pulp was then bleached with chlorine dioxide-sodium hydroxide-chlorine dioxide. The results are shown in Table 4.

Table 4

Kraft Pulp Bleaching B230

X at 60°C, 1 hr

Treatment	Bleached Pulp		
	Brightness (%)	Kappa Number	Yield (%)
(pH 5) DED (control)	78.4	2.21	98.8
(pH 5) XDED	79.4	2.04	98.9
(pH 7) DED (control)	77.4	2.35	98.1
(pH 7) XDED	81.6	1.74	97.6
(pH 9) DED (control)	76.8	2.45	99.4
(pH 9) XDED	81.4	1.75	98.7

D_1 - chlorine dioxide, 2.5% as chlorine, 70°C, 2 hr

D_2 - chlorine dioxide, 0.5% as chlorine, 70°C, 1 hr

E - sodium hydroxide, 1.5%, 50°C, 1 hr

X - xylanase treatment, pH 5, 7 or 9, 60°C, 1 hr

Kappa number is a measure of the amount of lignin in wood pulp. It is defined as the number of millilitres of 0.02M potassium permanganate solution which would be consumed by 1 gram of moisture-free pulp under AS 1301, APPITA P201 m-86, specified conditions.

It is evident from these results that bleaching in the presence of xylanase results in improved characteristics of brightness and kappa number, with yields comparable to that of the control. It is further evident that the xylanase gives optimal improvements in the alkaline pH range 7 to 9.

Example 11 Use of B230 as a Bleaching Aid - further example

The crude xylanase system (167nkat/g of pulp) was mixed with unbleached kraft pulp (35g oven dried basis) at 8% consistency and adjusted to pH 5,6,7,8,9 or 10 with appropriate buffer. The mixture was incubated for 1 hr at either 50,60 or 70°C. After the set time, the pulp was filtered to obtain a filtrate sample. The filtrate sample was briefly centrifuged and the absorbance at 456 nm was measured in a spectrometer. Absorbance units were converted to Pt-Co colour units from a standard graph where 500 colour units was obtained by dissolving K_2PtCl_6 (1.246g), $CoCl_2.6H_2O$ (1.00g) and HCl (100mL, 12M) in 1L of water. The colour units released from the pulp by the xylanase is a measure of the final bleach chemical savings. The optimal effective pH was found to be pH 7, independent of temperatures between 50 and 70°C (see figure 5).

Example 12

Our earlier International Patent Application PCT/AU95/00202 describes a xylanase-producing bacterium designated B698, which was isolated from wood compost, and which was deposited under the Budapest Treaty in the Australian Government Analytical Laboratories as Accession No. 94/7647.

The temperature profile, pH profile, thermal stability at 60°C and 65°C at different pH values, and bleach boosting activity of xylanases for B230 and B698 were compared, using the methods described above, and the results are summarised in Tables 5 to 9.

Table 5

Xylanase pH Profile

Birchwood xylan, 50°C

pH	Relative Xylanase Activity (%)	
	B698	B230
4	15	24
5	83	87
6	100	100
6.5	99	Not determined
7	77	72
8	69	76
9	37	32
10	11	11

Table 6

Xylanase Temperature Profile

Birchwood xylan, pH 6

Temperature	Relative Xylanase Activity (%)	
	B698	B230
40	65	45
50	85	63
60	100	100
65	Not determined	80
70	51	42
80	27	8

Table 7

Thermal Stability 60°C, 2 hrs

pH	Relative Xylanase Activity (%)	
	B698	B230
6	93	100
7	104	117
8	76	84
9	44	55

Note: At 60°C, pH 6, both B698 and B230 xylanases retained 90% of their activity after 22 hours.

Table 8

Thermal Stability 65°C, 2 hrs

pH	Relative Xylanase Activity (%)	
	B698	B230
6	95	71
7	103	48
8	30	7
9	9	0

Table 9

Kraft Pulp Colour Difference

60°C, 1 hr

pH	Colour Difference	
	B698	B230
5	247	371
6	832	995
7	1084	1038
8	943	921
9	967	991
10	195	283

In general, the properties of the xylanases from the two organisms are very similar. However,

1. In solution, B698 xylanase retains more activity over a wider temperature range at pH 6. B698 xylanase is clearly more thermally stable at 65°C over the pH range 6-9 than B230 xylanase.

2. In solution, at 50°C there is no differentiation between the two enzymes over the pH range 4-10.

3. On kraft pulp, at 60°C, both B698 xylanase and B230 xylanase are effective bleach boosting agents over the pH range 6-9.

4. On kraft pulp, at 70°C, B230 xylanase is more effective than B698 as a bleach xylanase boosting agent. This is a significant advantage.

5. During fermentation, bacterium B230 expresses more xylanase than bacterium B698 (11,000 nkat/ml and 7,000 nkat/ml respectively.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

CLAIMS

1. A bacterium, isolatable from wood compost, having the following characteristics:

A. Ability to grow at a temperature between 20° and 45°;

B. Ability to grow in the pH range of 5 to 9.5;

C. Ability to grow on Luria-Bertani agar at 37°;

D. Ability to grow under solid state or submerged culture conditions; and

E. Constitutive production and/or extracellular release of at least one protein with xylanase activity having an associated cellulase activity of less than 0.1%, wherein said at least one protein with xylanase activity has a molecular weight of about 28kD.

2. A bacterium according to Claim 1, which is a Gram positive, obligately aerobic rod-shaped spore-forming bacterium, in which the spores are centrally-located.

3. A bacterium according to Claim 1, or Claim 2 in which production of the at least one protein with xylanase activity is enhanced by growth in the presence of xylan, xylitol or of a lignocellulose substrate.

4. A bacterium according to Claim 1, Claim 2 or Claim 3 in which the at least one protein with xylanase activity has at least one characteristic selected from the group consisting of activity at about pH between 4.5 and 9.5, a thermal activity range up to 70°C, and high thermal stability up to 65°C.

5. A bacterium according to any one of Claims 1 to 4, in which the at least one protein with xylanase activity is effective on both soluble and insoluble xylans.

6. A bacterium having the characteristics of the bacterial isolate designated B230, as deposited in the Australian Government Analytical Laboratories under

Accession No. N94/41262, or a mutant or derivative thereof having the ability to produce a protein with xylanase activity as defined in any one of Claims 1 to 5.

7. A process for producing at least one protein with xylanase activity said process comprising cultivating a bacterium under conditions and for a time sufficient to produce said protein and collecting culture medium wherein said bacterium has the following characteristics:

A. Ability to grow at a temperature between 20 and 45°;

B. Ability to grow in the pH range of 5 to 9.5;

C. Ability to grow on Lauria-Bertani agar at 37°;

D. Ability to grow under solid state or submerged culture conditions; and

E. Constitutive production and/or extracellular release of at least one protein with xylanase activity, said protein having an associated cellulase activity of <0.1%.

8. The process of claim 7 wherein an amount of xylitol is added to said medium effective to increase production of said at least one protein.

9. An isolated preparation of a protein with xylanase activity said protein having an associated cellulase activity of ≤0.1% and a molecular weight of about 28kD as determined by SDS-PAGE.

10. The preparation of Claim 9 wherein said protein has at least one characteristic selected from the group consisting of activity at about pH between 4.5 and 9.5, a thermal activity range of up to 70°C, and high thermal activity at 65°C.

11. A xylanase having associated cellulase activity of less than 0.1%, and which is produced by a bacterium as defined in any one of Claims 1 to 6.

12. A bleaching aid, bleach booster or paper deinking composition comprising the protein according to Claim 9 or

the xylanase according to claim 11, together with an industrially acceptable stabiliser.

13. A process for bleaching kraft pulp, comprising the step of using the protein according to Claim 9 or the xylanase according to Claim 11 as a bleaching aid or bleach booster.

14. A process according to Claim 13, carried out at neutral to alkaline pH and/or at a temperature of 40° to 80°C.

15. A process according to Claim 14 carried out at a temperature of 50° to 70°C.

16. A process according to Claim 15, carried out at a temperature of 50° to 65°C.

17. A process for removal of printing ink from paper, comprising the step of using the protein according to Claim 9 or a xylanase according to claim 11.

18. A process for preparing an animal feed composition, comprising the step of adding the protein according to Claim 9 or the xylanase of Claim 11 to animal feed and forming said composition.

19. An animal feed composition comprising a protein according to Claim 9 or the xylanase of Claim 11.

20. A method of preparing dough for bread making which comprises the step of incorporating the protein according to Claim 9 or the xylanase of Claim 11 in said dough.

21. A dough for the preparation of bread, comprising the protein according to Claim 9 or the xylanase of Claim 11.

Fig 1 - B230 xylanase activity - pH profile at 50°C

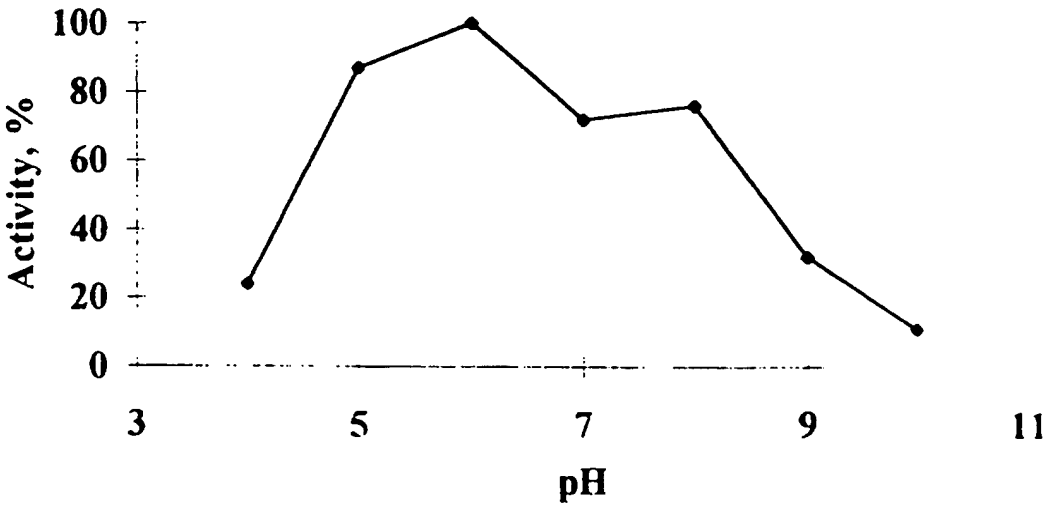


Fig 2 - B230 xylanase activity - Temperature profile at pH 6

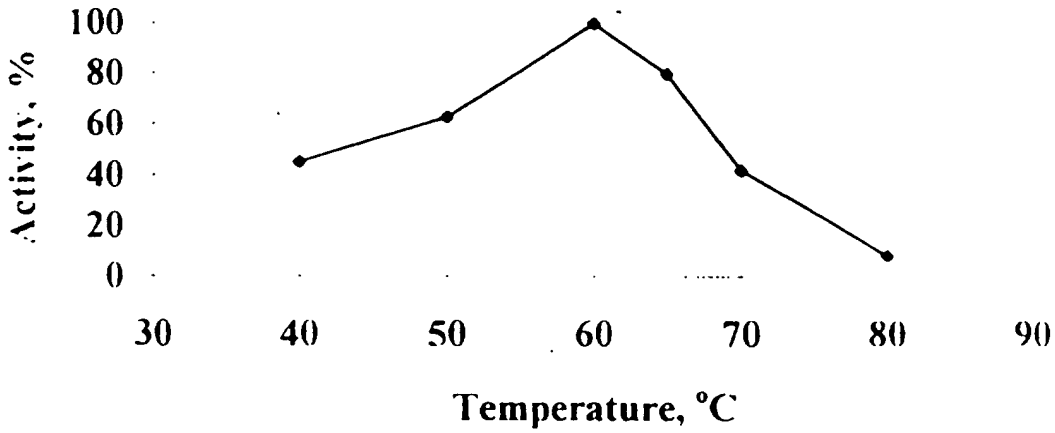
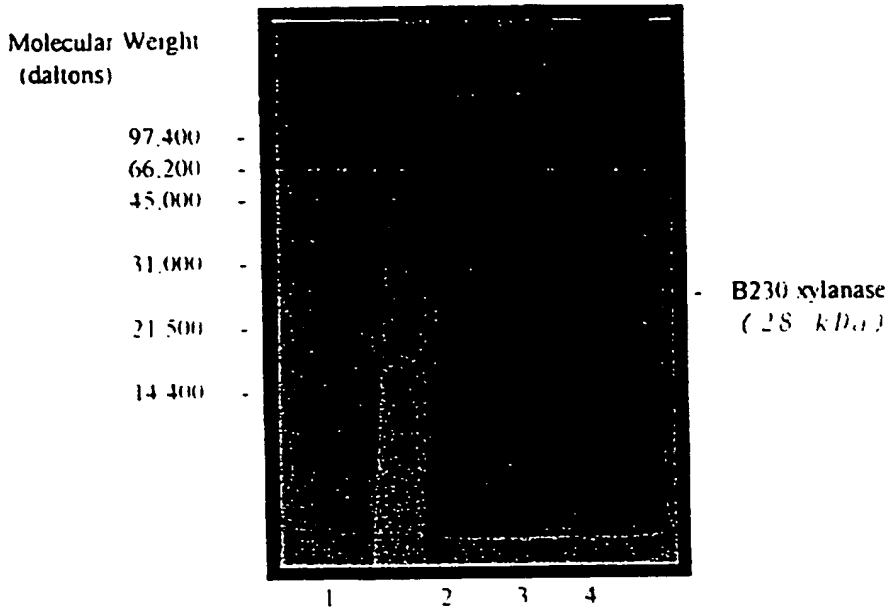
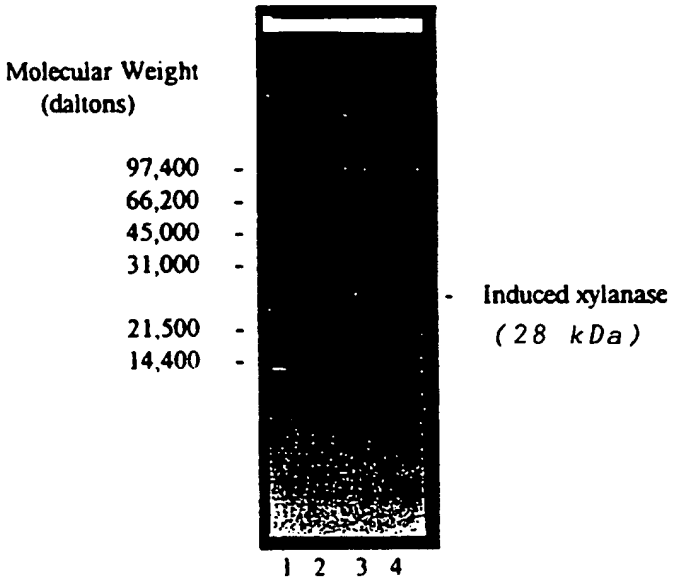


Figure 3



1 Protein molecular weight marker
2 B230 (HPLC fraction 11) 1638 nkat/ml.
3 B230 (HPLC fraction 12) 814 nkat/ml.
4 B230 (HPLC fraction 13) 164 nkat/ml.

Figure 4



1 B230 (xylanase induced, concentrated)
2 Protein molecular weight marker
3 B230 (xylanase uninduced)
4 B230 (xylanase induced)

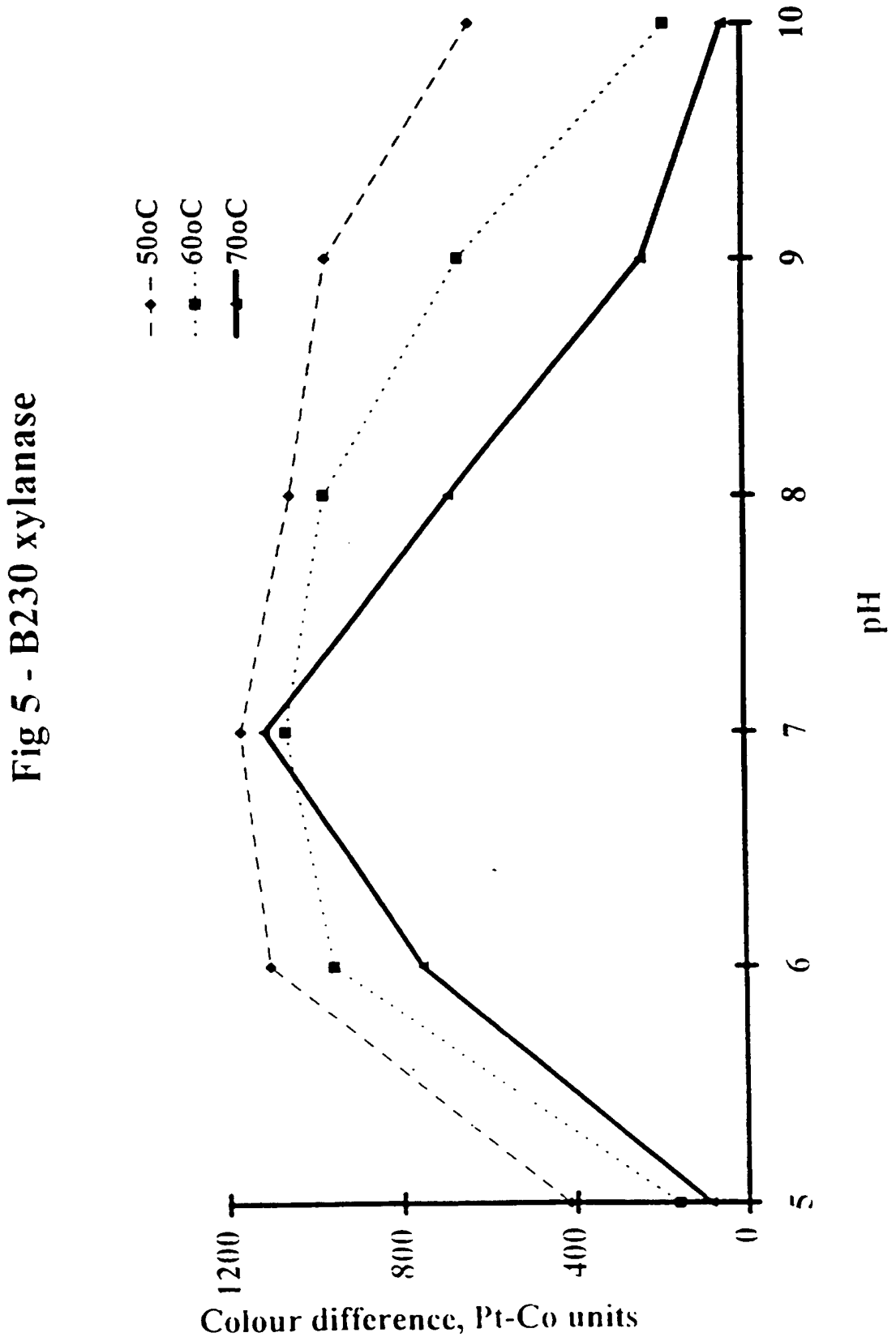


Fig 5 - B230 xylanase

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00709

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 1/20, 9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
DERWENT WORLD PATENT INDEX (WPAT),
CHEMICAL ABSTRACTS (CA - STN INTERNATIONAL)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
JAPAN PATENT INFORMATION ORGANIZATION (JAPIO)
USPM (DERWENT) AU: C12N 9/24

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT DATABASES (WPAT, USPM, JAPIO) KEYWORDS: XYLANASE(S) AND C12N/IC
CHEMICAL ABSTRACTS (CA - STN INTERNATIONAL) KEYWORDS: XYLANASE(S) AND (BACTERI? OR BACILLUS) AND 1990-1995 NOT PATENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9527779 (BIOTECH INTERNATIONAL LTD) published 19 October 1995. See whole document	7-9
A	Xylans and Xylanases Progress in Biotechnology volume 7 1992, Elvsevier Science Publications; et al, pages 325-337; Nissen A M et al "Xylanases for the Pulp and Paper Industry". see whole document	1-21

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 1996	19.02.1996

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00709

C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Canadian Journal of Microbiology (1993), Vol. 39, No. 12, pages 1162-1166 (A. BLANCO and F.I.J PASTOR) "Characterization of cellulase - free xylanases from the new isolated Bacillus sp. Strain BP-23". See whole document	1-21
A	The Journal of General Microbiology (1993), vol. 139, pages 1987-1993 (P. WANG et al.) "Xylanases from Streptomyces cyaneus : their production purification and characterization". See whole document	1-21
A	WO 94/04664 (NOVO NORDISK A/S) published 3 March 1994. See whole document	1-21
A	Agric Biol. Chem vol 49(7) pages 2033-2039 1985 (OKAZAKI W. et al) "Purification and Characterization of xylanases from alkalophilic themophilic Bacillus spp. See whole document	1-21
A	WO 92/03540 (NOVO NORDISK A/S) published 5 March 1992. See whole document	1-21
A	US 5306633 (GOTTSCHALK M. Et al) published 26 April 1994. See whole document	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 95/00709

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9527779	AU	21332/95				
WO	9203540	EP	489104	NO	920734	AU	63584/90
		BR	9007608	BR	9106781	CA	2090337
		EP	546045	FI	930804	NZ	239500
WO	9404664	DK	1055/92	EP	663949	FI	950852
US	5306633	DE	4226528	EP	585617	FI	933519

END OF ANNEX